Cytochrome c oxidase in *Neurospora crassa* contains myristic acid covalently linked to subunit 1

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ABSTRACT Radiolabel from [³H]myristic acid was incorporated by *Neurospora crassa* into the core catalytic subunit 1 of cytochrome c oxidase (EC 1.9.3.1), as indicated by immunoprecipitation. This modification of the subunit, which was specific for myristic acid, represents an uncommon type of myristoylation through an amide linkage at an internal lysine, rather than an N-terminal glycine. The [³H]myristate, which was chemically recovered from the radiolabeled subunit peptide, modified an invariant Lys-324, based upon analyses of proteolysis products. This myristoylated lysine is found within one of the predicted transmembrane helices of subunit 1 and could contribute to the environment of the active site of the enzyme. The myristate was identified by mass spectrometry as a component of mature subunit 1 of a catalytically active, purified enzyme. To our knowledge, fatty acylation of a mitochondrially synthesized inner-membrane protein has not been reported previously.

Cytochrome c oxidase (EC 1.9.3.1) is a multisubunit enzyme complex that catalyzes the terminal electron transfer and proton translocation steps of the mitochondrial electron transport system. In eukaryotes this complex, which is located in the inner mitochondrial membrane, contains a number of subunit peptides, the three largest of which are encoded by mitochondrial genes and synthesized on mitochondrial ribosomes, while the remainder are products of the nuclear genetic system and cytoplasmic protein synthesis (1, 2).

Subunits 1 and 2, the largest components of this enzyme, are the core catalytic subunits and they are common to oxidases in prokaryotes and eukaryotes. Subunit 1 of cytochrome c oxidase binds heme a and heme a_3 as well as one of the two copper groups of the complex, and it is the most conserved of all subunits in amino acid sequence. Hydropathy plots of subunit 1 sequences predict at least 12 α-helical transmembrane regions that contain the likely redox centers of the enzyme (3).

Earlier we observed (4, 5) that when we labeled *Neurospora crassa* cells with [¹⁴C]pantothenic acid, a labeled protein was produced that coimmunoprecipitated with the eight subunits of *N. crassa* cytochrome c oxidase. Since pantothenic acid is a substituent of the *N. crassa* mitochondrial acyl carrier protein (6), it seemed possible that this coimmunoprecipitation might indicate a functional relationship between cytochrome c oxidase and the acyl carrier protein, perhaps for transfer of an acyl moiety to modify one or more of the subunits. When we labeled *N. crassa* cells with [³H]myristic acid, we found that radioactivity was stably incorporated into about five proteins of isolated mitochondria (N.P.-V. and R.B., unpublished results). A subsequent report showed that animal cell mitochondria contained several unidentified proteins that were labeled *in vitro* with [³H]myristate or [³H]palmitate (7, 8). The NADH-cytochrome b_5 reductase, which is located at the outer mitochondrial membranes of animal cells, has been shown to be N-terminally modified with myristic acid (9, 10). These reports

prompted us to reexamine and extend our earlier findings, and in this report we identify one of these myristate-containing proteins in *Neurospora* mitochondria as subunit 1 of cytochrome c oxidase. We present evidence that this myristate is linked to an invariant lysine residue through an amide linkage. The acylation of several cellular and viral proteins with palmitic acid and myristic acid has been well characterized (11, 12), but, to our knowledge, there have been no previous reports of an identified mitochondrially synthesized inner-membrane protein being modified with a fatty acid.

MATERIALS AND METHODS

Growth, Radiolabeling, and Extraction of Cells. The conidia of *N. crassa* 74A were produced and germinated as described (13). When cells were labeled with tritiated fatty acids, we added cerulenin (Sigma, 1 μg/ml) 30 min before label to suppress biosynthesis of fatty acids (14). The cells were labeled, disrupted, and fractionated as described (15, 16).

Immunoprecipitation. Methods for immunoprecipitation analysis (16, 17), and SDS/polyacrylamide (12.5%) gel electrophoresis of proteins (18) have been published.

Hydroxylamine and Methanolic KOH Treatments. Samples of submitochondrial particles labeled with [³H]myristate or [³H]palmitate were treated with hydroxylamine (2 M, pH 9.9), with methanolic KOH (1 M in 20% methanol), or with Tris·HCl (1 M, pH 8.0), centrifuged at $100,000 \times g_{av}$ for 30 min, and subjected to SDS/polyacrylamide gel electrophoresis.

Proteolytic Digestion. We labeled cells with [³H]myristate, prepared submitochondrial particles for electrophoresis, and removed from the electrophoretic gel that region that contained subunit 1 of cytochrome c oxidase, guided by reversible staining (19) with Coomassie blue. The subunit protein was electroeluted from the gel and was denatured with urea (6 M). The urea concentrations were reduced to 2 M before addition of proteases (Boehringer Mannheim), Arg-C, Glu-C (or Staphylococcus V-8 protease), and Lys-C, and incubation at 37°C for 20 hr. CNBr was also used to digest the enzyme subunit after dissolving the freeze-dried protein in acetic acid and CNBr. Following electrophoresis of these digests, the gel lanes were sliced (100 1-mm slices) for analysis by liquid scintillation spectrometry (4).

Analysis of Fatty Acids. We separated subunit 1 of cytochrome c oxidase by electrophoresis. A fragment of the gel containing subunit 1 was excised and extracted with chloroform/methanol, and the proteins and associated fatty acids were subjected to acid hydrolysis (6 M HCl, 120°C, 18 hr) under vacuum followed by extraction with petroleum ether. The extracted material was dissolved in methanolic acetyl chloride (3% by volume) to methyl esterify the fatty acids. The samples were applied to KC18 reversed-phase thin-layer plates (Whatman) for ascending chromatography with acetonitrile/

Abbreviations: GC/MS, gas chromatography/mass spectrometry; myristate-me, palmitate-me, and stearate-me, methyl esters of fatty acids.
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acetic acid/water. We purified catalytically active cytochrome *c* oxidase of *Neurospora* (17) and subjected the gel-purified subunit 1 to acid hydrolysis and organic extraction, as above. Electron impact ionization mass spectra were acquired on a Kratos MS25 instrument with an ionizing energy of 70 eV, an accelerating potential of 4 kV, and ion source temperature of 210°C. Quantification was based upon summation of the characteristic fragment ions of $m/z = 74$ and 87, the two most intense fragments of saturated fatty acid methyl esters.

RESULTS

Incorporation of Radiolabel into Specific Mitochondrial Proteins. Cells of *Neurospora* were labeled with [³H]myristic acid or with [³H]palmitic acid, and mitochondria from these cells were isolated and the proteins were electrophoretically separated in a SDS/polyacrylamide gel. Fig. 1 shows that four or five proteins of these mitochondria incorporated the label from the [³H]myristate and at least two mitochondrial proteins incorporated the [³H]palmitate. Pretreatment of these cells with cerulenin, an inhibitor of fatty acid synthesis (14), enhanced incorporation of these labeled fatty acids into the same proteins.

We found that an antiserum to a mixture of all subunits of cytochrome *c* oxidase (17) precipitated one protein of about 42 kDa that had incorporated label from [³H]myristate (Fig. 2). Comparison with a cytochrome *c* oxidase immunoprecipitate, in which the subunits were labeled with [³⁵S]methionine, showed that the [³H]myristate-labeled protein migrated at the same position as subunit 1 (Fig. 3), a subunit encoded by the mitochondrial genome and synthesized within the mitochondrion. A similar analysis of [³H]palmitate-labeled mitochondrial proteins gave no indication that this fatty acid was present in any of the immunoprecipitated cytochrome *c* oxidase peptide subunits (data not shown). Reaction of these [³H]myristate-labeled proteins with either a preimmune antiserum or an antiserum against subunit 9 of the mitochondrial ATPase (16) precipitated no detectable radiolabel from this fatty acid (Fig. 2).

As another approach to test whether the labeling of subunit 1 of cytochrome *c* oxidase was specific to myristate rather than palmitate, we attempted to label the mitochondrial proteins in the presence of a 100-fold excess of either unlabeled palmitic acid or unlabeled myristic acid. We found that the unlabeled myristate blocked incorporation of [³H]myristate into the subunit 1 peptide, but the unlabeled palmitate did not (Fig. 3).

To learn if the myristoylation of subunit 1 could occur in a cell-free system, we isolated mitochondria from *Neurospora*
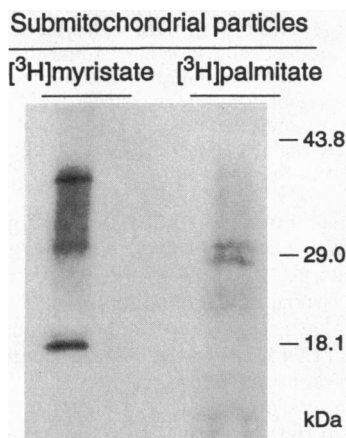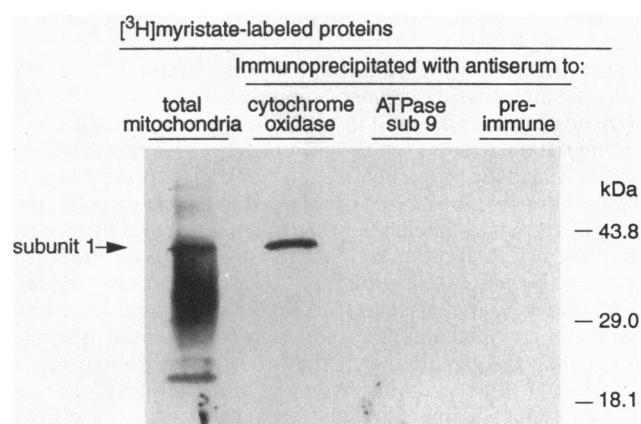
FIG. 2. Immunoprecipitation of [³H]myristate-labeled proteins from mitochondria of germinating spores with antisera prepared against *N. crassa* cytochrome *c* oxidase or the subunit 9 of the mitochondrial ATPase or a preimmune antiserum. The first electrophoretic lane shows the radiolabel in total mitochondrial protein, and the subsequent lanes show the results of electrophoresis of immunoprecipitates from lysates of the radiolabeled mitochondria.

cells and incubated these organelles with [³H]myristate. A 15-min incubation led to ATP-dependent labeling of a mitochondrial peptide of about 55.7 kDa that was immunoprecipitated with antiserum to cytochrome *c* oxidase (data not shown). This peptide had a slightly higher apparent molecular mass than the [³⁵S]methionine-labeled precursor (50.4 kDa) of subunit 1 (Fig. 3).

Characterization of the Chemical Linkage. We prepared submitochondrial particles from cells labeled with [³H]myristate to characterize the linkage between the myristate-derived radioactivity and the subunit 1 peptide. Extracts of these submitochondrial particles were treated with either hydroxylamine, methanolic KOH, or Tris·HCl buffer. The treatments with hydroxylamine and KOH should hydrolyze ester linkages but not amide linkages. The peptide identified as cytochrome *c* oxidase subunit 1 retained its [³H]myristate-derived label under these conditions, indicating that [³H]myristate-derived radioactivity is incorporated into the peptide in an amide linkage, as has been reported for most other cellular proteins that are modified with myristic acid. In parallel, we labeled cells with [³H]palmitate, prepared submitochondrial particles, and treated them with the same reagents, followed by electrophoresis. As expected for palmitic acid, whose characterized linkages to proteins are esterifications, this label was

FIG. 1. Incorporation of radiolabel from [³H]myristate and [³H]palmitate into mitochondrial membrane proteins. The germinating spores were radiolabeled with these fatty acids, and submitochondrial particles were prepared and subjected to electrophoresis in SDS/polyacrylamide gels.
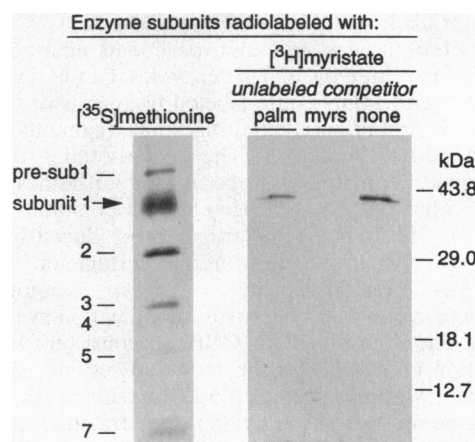
FIG. 3. Immunoprecipitation and electrophoresis of [³H]myristate-labeled cytochrome *c* oxidase subunit 1 from mitochondria of germinating spores labeled in the presence of 100-fold excess of unlabeled myristate or palmitate as competitors.

lost from the proteins after treatment with hydroxylamine or methanolic KOH.

Identification of Myristoylated Lysine Residue. To determine the amino acid residue of subunit 1 that was modified by myristic acid, we subjected the subunit 1 peptide, labeled with [^{3}H]myristate, to site-specific hydrolysis by three proteases and CNBr. Since the sequence of this subunit peptide from *N. crassa* is known (20–22), we expected that a proteolysis map of the [^{3}H]myristate-labeled peptide fragments, sized by electrophoresis, would allow us to identify a likely site for modification. The N terminus of subunit 1 of the cytochrome *c* oxidase of *N. crassa* has been sequenced at the amino acid level (20), and the N-terminal amino acid of the mature peptide is serine, rather than the glycine that is required for N-terminal myristoylation of other proteins. We expected, therefore, that the myristate must be linked to an internal amino acid, most likely via an amide linkage to lysine.

Glu-C (*Staphylococcus* V8) protease cuts at the carboxyl side of glutamic acid residues. Digestion of subunit 1 with this enzyme should yield four lysine-containing fragments (resolvable on our gel system) that range in size from 3.8 kDa to 26.4 kDa (see Fig. 5). The smallest fragment we found that contained label from [^{3}H]myristate was about 27 kDa (Fig. 4). The corresponding predicted fragment of 26.4 kDa (residues 249–485) contains four lysines that are candidates for modification, residues 271, 324, 404, and 416. Lys-C protease cuts at the carboxyl end of lysine residues and should produce seven resolvable lysine-containing fragments from subunit 1, ranging from 2.2 kDa to 17.9 kDa (Fig. 5). A major radioactive fragment of 5.7 kDa was obtained from digesting the [^{3}H]myristate-labeled subunit (Fig. 4); this labeled fragment likely corresponds to the predicted 5.9-kDa fragment (residues 272–324) that contains Lys-324. Digestion of subunit 1 with Arg-C protease, which cuts at the carboxyl end of arginine residues, should yield six resolvable lysine-containing fragments that range in size from 3.3 kDa to 11.9 kDa (Fig. 5). A major radioactive fragment of 3.0 kDa was derived from digestion of the [^{3}H]myristate-labeled subunit (Fig. 4). The corresponding 3.3-kDa fragment (residues 308–338) contains Lys-324. This proteolytic enzyme should yield other subunit 1 fragments that are only slightly larger (3.6–3.9 kDa), but none of these alternate predicted fragments contains a lysine residue that is included in the 26.4-kDa Glu-C fragment or in the 5.9-kDa Lys-C fragment.

One predicted lysine-containing peptide in each of the three digests is smaller than the minimum size of 1.6 kDa retained by the gels. However, each of these lysines was included within a resolvable proteolytic fragment (of an alternate digestion) that did not become labeled in these experiments. Therefore, they are not likely candidates for modification.

The results of these proteolysis experiments, analyzed in Fig. 5, show that the three proteolytic enzymes, Glu-C, Lys-C, and Arg-C, produce [^{3}H]myristate-labeled fragments of sizes that correspond to unique predicted digestion fragments of cytochrome *c* oxidase subunit 1. The corresponding predicted Lys-C fragment contains only one of the four lysines that are included within the Glu-C fragment; the common lysine is residue 324. The radiolabeled fragment produced by Arg-C digestion also corresponds to a predicted fragment that contains Lys-324. These three proteolytic digests, therefore, point to Lys-324 of subunit 1 as the site of modification by [^{3}H]myristic acid. The digestion with CNBr is consistent with this identification of Lys-324 as the modified residue, since this lysine resides within a predicted 5-kDa fragment that corresponds to the most prominent radioactive fragment produced by CNBr. However, the fragmentation pattern with CNBr was more complex and less clear than that produced by the proteolytic enzymes.

The formal possibility remains that the N-terminal amino acid of cytochrome *c* oxidase subunit 1 might be modified, even
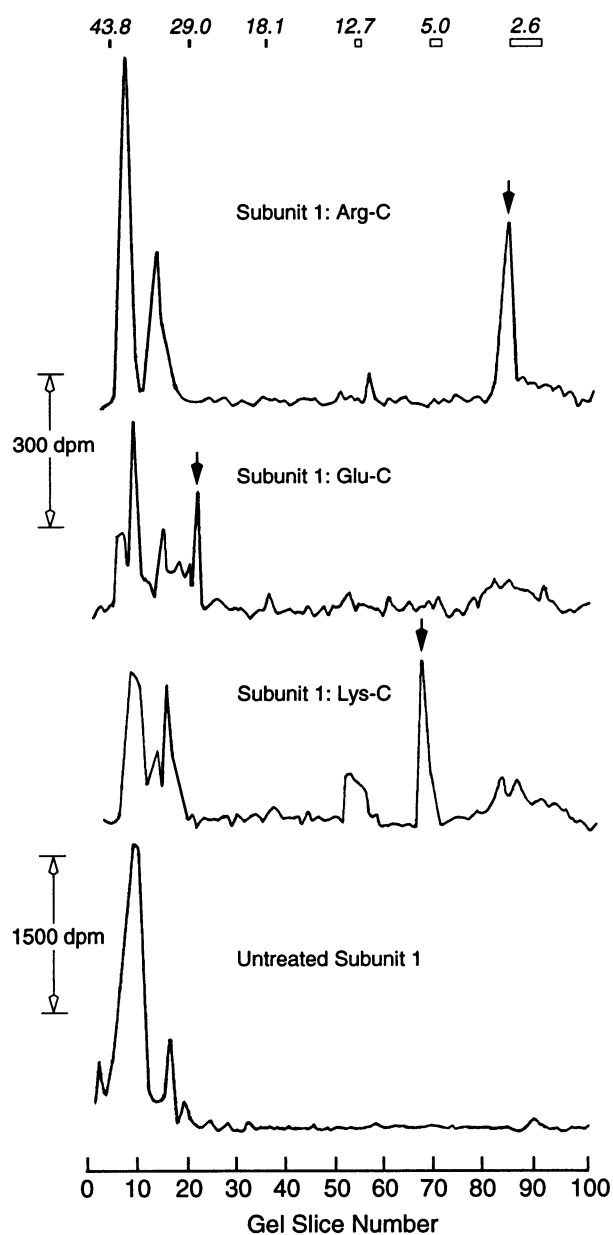

FIG. 4. Electrophoresis of [^{3}H]myristate-labeled subunit 1 of cytochrome *c* oxidase following proteolytic hydrolysis. The direction of electrophoresis was from left to right through cylindrical gels that were frozen and sliced mechanically. Individual 1-mm slices of the electrophoretic gel were analyzed by liquid scintillation spectrometry and plotted. Proteolytic agents were the enzymes Arg-C, Glu-C, and Lys-C. Molecular masses are indicated in kDa. The 300-dpm scale applies to the upper three electropherograms, and the 1500-dpm scale applies to the electrophoresis of the uncut subunit 1.

if it is an amino acid other than the canonical glycine residue. However, the results from our experiments argue against this possibility. Although two of the proteases, Glu-C and Arg-C, would produce N-terminal fragments that are too small to be resolved by our gels, the N-terminal fragment produced by Lys-C digestion (1.94 kDa for the mature subunit 1) is within the limits of gel resolution, and no radiolabeled fragment of the appropriate size was produced.

Demonstration that Myristate Is a Component of Subunit 1. Evidence presented above demonstrates that label from [^{3}H]myristate is incorporated into subunit 1 of the *Neurospora* cytochrome *c* oxidase. To test whether myristate itself is chemically incorporated into the subunit peptide, we prepared subunit 1 from submitochondrial particles of cells labeled with
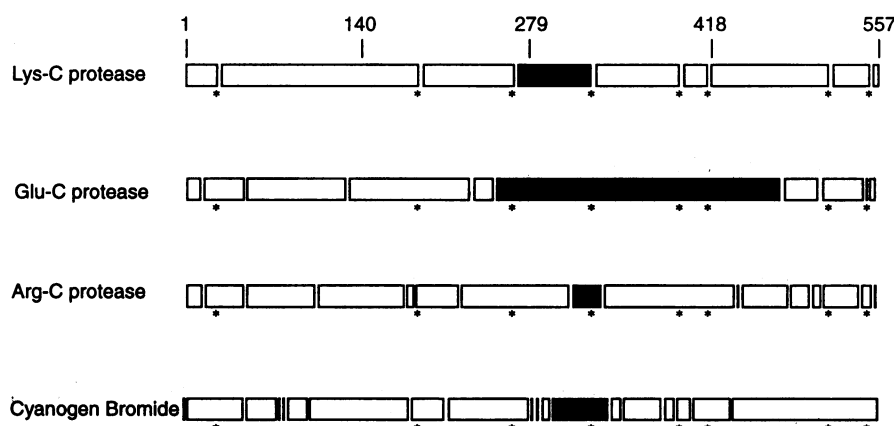
FIG. 5. Analysis of proteolytic digestion of subunit 1 radiolabeled with [3H]myristic acid, showing the deduced lysine residue modified by this fatty acid. Proteolytic agents were the enzymes Arg-C, Glu-C, and Lys C or CNBr. Asterisks denote lysine residues. The boxes indicate proteolysis fragments generated by each of these agents, and filled boxes denote unique peptides in each digest that contained radioactivity from [3H]myristic acid and that contained the common Lys-324 residue modified with myristic acid.

[3H]myristate, subjected this protein to acid hydrolysis, and extracted the petroleum ether-soluble fraction. This fraction was subjected to methyl esterification and to chromatography on reversed-phase KC_{18} plates that permitted separation of palmitate-me and myristate-me. Guided by cochromatography with authentic esters of the fatty acids, we removed 1-cm^2 zones of the plates that would contain esters of myristate (R_f = 0.190) or palmitate (R_f = 0.109) for analysis by liquid scintillation spectrometry. We found that the majority of the radioactivity recovered was chromatographically identical to methyl-esterified myristic acid. Protein containing about 20,000 dpm from [3H]myristate was originally subjected to acid hydrolysis, and following organic extraction of this hydrolysate and chromatography, we recovered about 16,000 dpm in the leading zone as myristate-me, 2400 dpm in the trailing zone as palmitate-me, and 1500 dpm remaining at the origin. The control electrophoretic gel fragment, a region containing no protein, yielded background radioactivity of 260 dpm, 180 dpm, and 150 dpm, respectively, for these three chromatographic fractions.

We asked if the myristate-modified subunit 1 was perhaps an intermediate in the enzyme subunit assembly pathway or if the myristate modified the subunit of the assembled enzyme. We purified catalytically active cytochrome c oxidase from Neurospora (17) and separated the subunits by electrophoresis in SDS gels. We recovered subunit 1, along with subunit 2 and a blank gel fragment as controls, for acid hydrolysis and organic extraction of the fatty acids. This fraction was subjected to methyl esterification and analyzed by gas chromatography/mass spectroscopy (GC/MS). The subunit 1 peptide contained >25 times the amount of myristate-me found in an enzyme subunit 2 control sample excised from the same gel. The trace amounts of palmitate-me and stearate-me (in comparison to myristate-me) found in the subunit 1 fraction were comparable to the two control samples. The sample containing subunit 1 yielded a summed-peak height signal equivalent to 21.4 ng of myristate-me, whereas this same fraction contained 3.7 ng and 2.4 ng of palmitate-me and stearate-me, respectively, as determined by comparison to quantitative standards. Control samples, either subunit 2 or an empty zone of the gel, respectively, contained 0.8 ng or 1.2 ng of myristate-me, 2.9 ng or 5.3 ng of palmitate-me, and 3 ng or 3.4 ng of stearate-me. The results thus clearly identify myristic acid as a component of the purified subunit 1 of the active enzyme.

DISCUSSION

We learned that several proteins in mitochondria of N. crassa incorporate radioactivity in vivo from [3H]myristate. These modified proteins all appear to be associated with mitochondrial membranes, since they are retained in submitochondrial particles. We identified one of the major myristoylated proteins as subunit 1 of cytochrome c oxidase, and we found that myristate was linked to the subunit by an amide bond. There is strong evidence that the modification occurs on an internal lysine, residue 324. This lysine is extremely well conserved across phylogenetic groups, being invariant in 50 representative sequences that we examined from the Swiss Protein data base. The lysine residue is within one of the transmembrane domains of subunit 1, in a region that has not been known to be involved in the heme- and copper-binding functions of the subunit (3). We found that the mature subunit 1 of the active enzyme contains the myristate modification.

Covalent modification with myristate has been reported for several eukaryotic cellular and viral proteins (11, 12). The prevalent type of protein myristoylation, which has been well characterized, occurs cotranslationally at the α-amino group of the N-terminal glycine (position 2). This N-terminal myristoylation is specific for myristic acid and has stringent sequence requirements with respect to the five amino acids that follow glycine (23). The myristoyl CoA:N-myristoyl transferase of Saccharomyces cerevisiae has been isolated and characterized (24). This transferase is a cytoplasmic protein that is not found associated with membranes or organelles (25).

There are only a few examples of internal modifications specifically with myristate. The precursor to the α tumor necrosis factor is modified at an internal lysine (26), and the insulin receptor (27), the μ immunoglobulin heavy chain (28), and the interleukin 1 α and β precursors are also modified internally by this fatty acid (29). The requirements and mechanism for internal myristoylation of cytoplasmic proteins have not been characterized. The myristoylation described in the present report must occur within the mitochondria, since the cytochrome c oxidase subunit 1 is encoded by a mitochondrial gene and synthesized within the organelle. This suggests that the mitochondria may contain a distinct system for modifying proteins with myristic acid. It is not known whether this organellar modification involves the mitochondrial acyl carrier protein. However, it has been determined that the amide-linked acylation of prohemolysin by pathogenic Escherichia coli is mediated by the bacterial acyl carrier protein and that acyl-CoA is ineffective in this transfer (30). We earlier reported (4, 5) that cytochrome c oxidase of N. crassa coimmunoprecipitated with a pantothenate-containing moiety that now appears to be the acyl carrier protein (6). More recently it has been learned that the mitochondrial NADH dehydrogenase (complex I) of N. crassa (31) and of bovine heart muscle (32)

contains a subunit peptide that is the acyl carrier protein. A gene disruption in *Saccharomyces*, leading to mitochondrial respiratory deficiency, was recently reported (33), and the disrupted gene encodes a sequence that strongly resembles the condensing enzyme of fatty acid synthesis. There were no major changes in mitochondrial membrane lipids in the mutant strain. However, the myristic acid level correlated with the gene defect, showing a severalfold increase upon complementation of the mutant and restoration of respiratory competency.

What might be the function of myristate modification of subunit 1 of cytochrome *c* oxidase in *N. crassa*? It seems unlikely that this modification is required for integration of the protein into the lipid bilayer of the inner mitochondrial membrane, since the unmodified subunit 1 is extremely hydrophobic, with numerous predicted transmembrane α-helices, one of which contains the likely site of myristoylation. Protein myristoylation also mediates protein–protein interactions, and it is possible that subunit 1 acylation contributes to the assembly with subunit 2, forming the core of the complex, and perhaps with other subunits of this enzyme complex. Earlier we showed that cells of a pantothenate-deficient auxotroph of *N. crassa* were unable to completely assemble the subunits of cytochrome *c* oxidase into a functional complex in the absence of this cofactor, even when they were supplemented with fatty acids (5). However, it is not known if this oxidase assembly defect is related to a cellular inability of the auxotroph to transfer myristate to subunit 1.

The structurally simpler bacterial oxidases, which have strong homology to the mitochondrial cytochrome *c* oxidase, recently have been reexamined with techniques of site-directed mutagenesis and improved physical methodologies to gain new insight into the structure of the active site of the enzymes (34). Among several residues whose substitutions disrupted the structure and function of subunit 1 of the *Rhodobacter sphaeroides* cytochrome aa_3 was Lys-362, which is homologous to Lys-324 of *N. crassa* subunit 1. Substituting a methionine residue for this lysine affected the axial ligation of heme a_3, a change that was associated with loss of catalytic activity and altered CO binding. Replacement of Lys-362 with arginine also led to a catalytically inactive enzyme, and similar results were obtained with Lys-362 replacement in a parallel study of cytochrome *bo* of *E. coli*, although without the effect on heme *a* spectra. These results led to the proposal that Lys-362 and nearby residues are important for delivering substrate protons to the site where oxygen is reduced to water and/or for providing a channel for the water to leave the oxidase enzyme. This channel may also transport protons across the membrane. This report by Hosler *et al.* (34) is the first, of which we are aware, that analyzes the role of this conserved lysine in the activity of cytochrome *c* oxidase subunit 1. Our present finding that this lysine is myristoylated in the *Neurospora* enzyme suggests that the fatty acylation itself could be required for a functional catalytic site.

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